EXHIBIT B

xviruses as expression vectors

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*Pox*viruses are widely used for the cytoplasmic expression of recombinant genes in mammalian cells. Recent improvements allow high expression and simplify the integration of multiple foreign genes. Vaccinia virus mutants and other poxviruses that undergo abortive infection in mammalian cells are receiving special attention because of their diminished cytopathic effects and increased safety. New replicating and 'non-replicating' vectors, encoding the bacteriophage T7 RNA polymerase for transcription of recombinant genes, have been engineered.

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Abbreviations

β-gal	β-galactosidase
gpt	xanthine-guanine phosphoribosyltransferase
gus	β-glucuronidase A gene
MVA	modified vaccinia virus Ankara
rVV	recombinant VV
VV	vaccinia virus

Introduction

The purpose of this review is to highlight recent progress in the construction and use of poxvirus vectors; earlier studies are described and referenced elsewhere [1•–3•]. Recombinant gene expression by vaccinia virus (VV), the prototypic member of the orthopoxvirus genus of the Poxviridae family, was first demonstrated in 1982 [4,5]. Special features of the VV vector system include a capacity to stably integrate over 25,000 base pairs of foreign DNA into the viral genome without loss of infectivity, a cytoplasmic site of gene expression, and a wide vertebrate host range. Other poxviruses, including members of the avipoxvirus genus, however, have greater host specificity. The versatility, for both transient and stable expression, was extended by the incorporation of bacteriophage transcriptional systems into the vaccinia virus genome. Poxvirus expression systems have been used for analysis of protein structure/function relationships, protein processing and intracellular trafficking, antigen presentation, the determinants of cellular and humoral immunity, and as live recombinant vaccines.

Construction of recombinant vaccinia virus by homologous recombination

The original and still most widely used method of producing recombinant VV (rVV) is by homologous recombination. Cells are transfected with a transfer plasmid, which contains the recombinant gene under control of a VV promoter flanked by several hundred base pairs of VV derived DNA, and infected with VV. Recombination occurs between homologous sequences in the plasmid and viral genome. A variety of methods are available for the isolation of rVV including selection based on bromo deoxyuridine or antibiotic-resistance, detection of a reporter gene expressing a colour marker, complementation of a host range or small plaque phenotype, and direct antibody staining of plaques or DNA hybridization [6]. The *E. coli* β-glucuronidase A gene (*gus*), provides an alternative to β-galactosidase (β-gal) as a colour marker [7,8]. *gus* is a relatively small gene and fortuitously lacks commonly used restriction sites, thereby simplifying vector constructions. Furthermore, as the substrates for *gus* and β-gal are not cross-reactive, both reporter genes can be used for making rVV with multiple inserts. A *gus*–neo cassette provides both colour detection and antibiotic-resistance (T Shors, B Moss, unpublished data). A convenient *E. coli* xanthine-guanine phosphoribosyltransferase (*gpt*)–*gus* fusion protein has been described for similar use [9]. The reporter gene encoding green fluorescent protein has been incorporated into VV [10]. A particularly efficient selection method, based on the repair of the non-plaque-forming phenotype of a mutant VV, neither interrupts a vaccinia virus gene nor inserts a reporter or antibiotic selection marker [11].

The stable integration of a selectable marker precludes its use for selection of a second gene; in addition, extra genetic material may not be desirable in a rVV that is to be used for clinical trials. Schemes in which antibiotic-resistance or colour marker genes are integrated and then spontaneously deleted by recombination have been developed [12–14]. A modified procedure uses both *gpt* and β-gal for transient selection (F Scheiflinger, F Dorner, FG Falkner, personal communication). An alternative protocol, in which a separate non-integrating plasmid containing *gpt* is cotransfected with the transfer plasmid provides a several-fold enrichment of rVV by antibiotic selection [15].

Construction of rVV by *in vitro* ligation

The *in vitro* ligation of a foreign gene into the VV genome provides an alternative to homologous recombination [1•]. Since VV DNA is not infectious, the cells are transfected with rVV DNA and infected with a host-restricted helper

avipoxvirus or conditionally lethal VV. These techniques allow the efficient insertion of very large DNA fragments or even libraries of DNA fragments directly into the vaccinia virus genome. rVV genomes have been engineered with promoters and unique restriction endonuclease sites to facilitate cloning and expression [16,17].

Poxvirus promoters

Viral promoters are required for expression of foreign genes because poxviruses replicate in the cytoplasm and have their own transcriptional system. The promoters are about 30 base pairs long and come in three varieties (early, intermediate and late) and various strengths. Early promoters are expressed before viral DNA replication, whereas intermediate and late promoters are expressed successively after DNA replication [18•]. Despite their lower intrinsic activity, early promoters may be preferred in order to obtain expression before virus-induced cytotoxic effects and for cells that are not fully permissive. To achieve both early and high expression, many transfer vectors contain natural, modified or strong synthetic tandem early/late promoters [19•,20].

Replication-deficient vaccinia virus expression systems

VV is infectious and therefore use of a containment facility with a microbial safety cabinet and vaccination of laboratory workers may be recommended or required by some institutions. Two highly attenuated strains of vaccinia virus, NYVAC and modified vaccinia virus Ankara (MVA), have been approved by the National Institutes of Health (USA) intramural biosafety committee for use without a safety cabinet or vaccination. NYVAC was constructed by deleting 18 genes, some of which are involved in host-range and pathogenesis [2•]. In human cell lines, replication of NYVAC is blocked at an early stage, whereas productive infection is observed in African green monkey kidney (VERO) cells and chick embryo fibroblasts. MVA, by contrast, is blocked in virion assembly; consequently, late expression of recombinant proteins occurs even in non-permissive cells [1•]. MVA grows to high titers in chick embryo fibroblast and, surprisingly, in baby hamster kidney 21 cells but replicates only slightly or not at all in human and other mammalian cell lines tested because of multiple gene defects (MW Carroll, B Moss, abstract W41-4, 16th annual meeting of the American Society for Virology, Bozeman, MO, July 1997).

Recently, a mutated VV with a deleted uracil DNA glycosylase gene was constructed using a complementing cell line [21••]. The enzyme is required for DNA replication, so that prolonged early gene expression occurs in non-permissive cells. This defective virus may be useful as an expression vector. Controlled cross-linking of VV DNA, with psoralen and *UV* light, severely reduces viral infectivity while allowing expression of a recombinant gene regulated by a viral early promoter [22]. Under appropriate conditions, virus-induced cytotoxicity and inhibition of host protein synthesis were reduced while recombinant gene expression was prolonged. Restrictions of the latter system include poor expression of genes with large *UV* target sizes and inability to employ late promoters for high expression.

Bacteriophage/vaccinia virus hybrid expression systems

The hybrid system is based on the integration of a bacteriophage RNA polymerase gene into the genome of VV for the specific expression of recombinant genes. Transient and stable forms of the expression system are available [1•]. In the widely used transient system, cells are infected with a rVV that expresses the bacteriophage T7 or bacteriophage SP6 RNA polymerase and are transfected with a plasmid that contains a gene under the control of a T7 or SP6 promoter. High expression is achieved when the encephalomyocarditis virus cap-independent ribosome binding site is present to enhance translation. In the stable form of the system, the T7 promoter-regulated gene is also incorporated into the VV genome. Recent modifications of this system provide stringent chemical or thermal inducibility of recombinant gene expression [23]. Another modification incorporates the cowpox virus host range gene into rVV, allowing efficient gene expression in normally nonpermissive Chinese hamster ovary cells [24]. The T7 RNA polymerase gene has been incorporated into the MVA strain of VV, allowing its use for transient gene expression in a defective vector system [25,26].

Other poxviruses as vectors

Poxvirus promoters and transcription factors are conserved among even distantly related poxviruses, so that the basic strategies developed for VV expression vectors can be employed for all members of the family. Host-specificity has been the major inducement leading to the development of alternative poxvirus vectors. The orthopoxviruses, raccoonpox [27] and ectromelia (mousepox) [28] may be useful vectors for wild-life vaccines or for basic studies of pathogenesis and immunity in the laboratory mouse, respectively. Members of the capripoxvirus and leporipox genera have been developed as vectors for cattle [29] and rabbits [30], respectively. Avipoxviruses, initially developed as host-range restricted vaccines for birds, have also been promoted as human vaccine candidates because of their safety and lack of immunological cross-reactivity with vaccinia virus [2•]. The stage at which fowlpox virus replication is blocked may vary for different non-avian cell lines, and both early and some late gene expression have been detected [31]. A recombinant fowlpox virus expressing the T7 RNA polymerase gene offers an alternative to rVV for the transient expression of recombinant genes [32].

Basic laboratory applications of poxvirus vectors

Investigators continually find novel and important uses for rVV vectors, so only a few recent examples are

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